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The selective cyclooxygenase-2 inhibitor mavacoxib (Trocoxcil™) exerts anti-tumour effects *in-vitro* independent of cyclooxygenase-2 expression levels

Short running title:

The anti-tumour effects of mavacoxib

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Abstract

The inducible inflammatory enzyme cyclooxygenase-2 (COX-2) and its product prostaglandin E2 (PGE_2) are prominent tumour promoters, and expression of COX-2 is elevated in a number of tumours of both humans and canines. Targeting COX-2 in cancer is an attractive option due to readily available non-steroidal anti-inflammatory drugs (NSAIDs), and there is a clear epidemiological link between NSAID use and cancer risk. In this study we aim to establish the anti-tumourigenic effects of the selective, long-acting COX-2 inhibitor mavacoxib. We demonstrate here that mavacoxib is cytotoxic to a panel of human and canine osteosarcoma, mammary and bladder carcinoma cancer cell lines; that it can induce apoptosis and inhibit the migration of these cells. Interestingly, we establish that

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mavacoxib can exert these effects independently of elevated COX-2 expression. This study highlights the potentially novel use of mavacoxib as a cancer therapeutic, suggesting that mavacoxib may be an effective anti-cancer agent independent of tumour COX-2 expression.

Keywords: canine; comparative oncology; COX-2; COX-2 independent effects; mavacoxib

Introduction

The cyclooxygenase (COX) enzymes (COX-1 and COX-2) are key in the synthesis of prostaglandins, prostacyclin and thromboxane from arachidonic acid. COX-2 is an inducible enzyme stimulated as part of an immune response by inflammatory cytokines and growth factors, and produces inflammatory prostaglandins involved in pain signalling and swelling [1]. In cancer, COX-2 is overexpressed with adverse consequences; tumours with elevated COX-2 levels are more aggressive, with increased incidence of metastasis and resistance to therapeutics. Many cancer types (both human and canine) have increased expression of COX-2, including breast [2–5], lung [2,6], skin [7], colon [2,8,9] and bone cancer [10–13]. The COX-2 / prostaglandin axis targets many downstream factors that play important roles in tumourigenic pathways. Prostaglandins, particularly PGE_2, are involved in enhancing tumour growth and progression through increasing cancer cell proliferation [11,14], increasing the metastatic and invasive potential of cancer cells via increased mobility [15,16] and enhancing angiogenesis via stimulation of vascular endothelial growth factor (VEGF) [17]. PGE_2 signalling mechanisms are also involved in the ability of cancer cells to evade apoptosis. This occurs due to the deregulation of apoptotic proteins via the loss, damage or inhibition of the tumour suppressor protein p53, and by increasing the expression of anti-apoptotic proteins and reducing the expression of pro-apoptotic proteins [15]. The ability to avoid controlled cell death allows damaged cancer cells to grow rapidly to form an aggressive tumour.

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the COX enzymes, and coxibs are a family of NSAIDs which selectively target COX-2. The use of NSAIDs to treat inflammatory conditions such as arthritis is universally accepted. Experimentally, both NSAIDs and coxibs have been shown to inhibit tumorigenesis [8,18–20] by inhibiting cancer cell growth and proliferation [14,21], modulating apoptotic activity [22–24], reducing the metastatic and invasive potential of cells [16,25,26] and by inhibiting angiogenesis [27–29]. Positive results from pre-clinical and clinical trials in which coxibs have been used as a preventative agent or as a therapeutic in combination with either chemotherapeutics or surgery demonstrates the potential of using such drugs as cancer therapeutics [17,30–33]. In veterinary medicine mavacoxib (Trocoxil™) is of interest because it is currently licensed to treat pain and inflammation associated with canine osteoarthritis where long term (up to 6 months) treatment is required [34]. Mavacoxib is unique in the family of NSAIDs and coxibs as it exhibits a low clearance rate, a relatively large distribution volume and a long plasma half-life, resulting in a reduced dosing regimen. Consequently, the potential clinical benefit of using mavacoxib is high.

There is limited research into the potential anti-cancer effects of mavacoxib. We have previously shown that mavacoxib exhibits anti-proliferative and pro-apoptotic effects in a panel of canine cancer cell lines and cancer stem cells *in-vitro* [35,36]. Here, we investigate the effects of mavacoxib on a panel of canine and human cancer cell lines. The cancers studied include osteosarcoma, mammary carcinoma and transitional cell carcinoma (TCC) of the urinary bladder, which exhibit cross species similarities. COX-2 has an important role in disease progression in these cancer types and in both species the use of NSAIDs has shown positive therapeutic effects [2,4-5,11,20,35,37–48]. We aim to determine if mavacoxib is directly cytotoxic to, and is able to inhibit the migration of these particular cell lines. We aim to begin

to establish not only if mavacoxib has potential as an anti-cancer agent against these cancer types, but also if this response is comparable between species.

Materials and methods

Cell culture

The canine osteosarcoma cell lines, KTOSA5 [35] and CSKOS [49] (gifted from Chand Khanna (NIH)) were maintained in Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher Scientific, Massachusetts, USA). The human osteosarcoma cell line U2OS was grown in McCoys 5A modified medium (ThermoFisher Scientific). Canine mammary carcinoma cells REM134 (REM) [50] were maintained in DMEM. Canine inflammatory mammary carcinoma cells LILY (gifted from Dr De Maria Raffaella, University of Turin) were grown in RPMI medium 1640 (ThermoFisher Scientific) supplemented with 10 μg/mL human insulin (Sigma-Aldrich, Missouri, USA) and 10 ng/mL human recombinant EGF (Peprotech, London, UK). Canine transitional cell carcinoma of the urinary bladder cell lines K9TCC, K9TCC-AXA, K9TCC-In and K9TCC-Sh (gifted from Deborah Knapp and Jane Stewart (Purdue University)) were maintained in DMEM/F-12 (ThermoFisher Scientific) containing HEPES and L-glutamine. Human transitional cell carcinoma of the urinary bladder cell lines T24, 5637 and HT-1376 cells were gifted from Anne Kiltie (Oxford University). The T24 cell line were maintained in McCoy's 5A (modified) medium, 5637 cell line were maintained in RPMI-1640 medium (ATCC modification) (ThermoFisher Scientific) and the HT-1376 cell line were maintained in Advanced MEM (minimum essential medium) (ThermoFisher Scientific) supplemented with 1% glutamine. All cell culture medium was supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin/streptomycin (ThermoFisher Scientific) and were maintained at 37°C in a humidified 5 % CO_2 incubator.

Cytotoxic drug treatment

Mavacoxib (Trocoxcil™, Zoetis, London, UK) was dissolved in DMSO and diluted in media immediately before use. Vehicle controls were included in all experiments.

Cell viability assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used according the manufacturer's instructions. Briefly, 500 cells per well were seeded in a 96-well plate in triplicate. Cells were incubated for 24 hours at 37°C, 5% CO_2. Serial dilutions of mavacoxib (0 – 200 μM) were added and cell viability was assayed after 72 hours. Luminescence was recorded by a spectrophotometer (WPA Biowave, Biochrom, Cambridge, UK) using the Viktor3 software (PerkinElmer, Massachusetts, USA). Data was averaged and normalized against the average signal of vehicle control treated samples and a dose-response curve was produced. The IC50 for each cell line treated with mavacoxib was calculated using GraphPad Prism version 7.0c for Mac OS X (GraphPad software, CA USA) using the log(inhibitor) vs response variable slope equation.

Colony formation assay

Cells were seeded as single cells at low density (250 cells / 10 cm plate for CSKOS, REM, K9TCC, K9TCC-AXA, K9TCC-In, K9TCC-Sh cells and 100 cells / 10 cm plate for KTOSA5 cells) and treated with 0, 25, 50 or 75 μM mavacoxib while still in suspension. Plates were incubated at 37°C in humidified 5% CO_2 incubator for 10-14 days. Cell culture media was changed once a week. Colonies were fixed by incubating with methanol for 5

minutes at room temperature and stained with Giemsa stain at 1:20 dilution (ThermoFisher Scientific). For each concentration tested, plates were set up in triplicate or quadruplicate. The total number of colonies was manually counted and the average number of colonies for each condition calculated.

Cellular migration assay

Scratch assay. Cells were grown until 80-90% confluent in 10 cm plates. The cell culture media was replaced and plates were treated with 0 – 125 μM mavacoxib and incubated at 37°C in humidified 5% CO_2 incubator overnight. A scratch was made in the confluent monolayer of cells down the centre of each plate with a pipette tip.

Migration assay. Cells were seeded at 3 x 10^5 cells / well of the cell culture insert in a volume of 70 μl cell culture medium. Plates were incubated for 24 hours at 37°C, 5% CO_2. Once confluent, cell culture medium was removed from each well and replaced with new cell culture medium containing 0 – 125 μM mavacoxib and incubated at 37°C, 5% CO_2 for 24 hours. After 24 hours incubation each insert was removed from its well. One ml of cell culture medium was added to each well.

In both assays the width of the gap was measured at 6 points using the Axiovert 40 CFL microscope coupled with an AxioCAM HRm camera (Carl Zeiss Ltd, Cambridge, UK) and an average taken. Measurements were made at 0, 4, 8, 24, 28, 32 and 48 hours. Scratch width was converted to 'relative migration distance' of cells, where the distance is a percentage of the initial wound width at 0 hours.

Apoptosis assay

Apoptotic activity was measured using the Caspase-Glo® 3-7 Assay (Promega) according to the manufacturer's instruction. Briefly, cells were seeded at 500 cells per well in 96-well plates and incubated for 24 hours at 37°C, 5% CO_2. Serial dilutions of mavacoxib (0 – 200 μM) were added to appropriate wells. Apoptotic activity was measured 48 hours post-treatment. Luminescence was recorded by a spectrophotometer (WPA Biowave, Biochrom) using the Viktor3 software (PerkinElmer). Luminescence is proportional to the amount of caspase activity present. Data was averaged and normalized against the average signal of vehicle control treated samples.

PGE_2 ELISA

The Canine Prostaglandin E2 ELISA Kit from BlueGene (AMS Biotechnology, Abingdon, UK) was used to determine the amount of PGE_2 exported from the cells into the cell culture media. Cells were seeded in equal numbers using the relevant cell culture media which was replaced with fresh cell culture media when the cells reached 50% confluency. After 24 hours incubation the cell culture media was removed from each flask, centrifuged and the ELISA carried out following the manufacturer's protocol. Optical density of the samples was measured at 450 nm using the spectrophotometer (WPA Biowave, Biochrom) with Viktor3 software (PerkinElmer). A standard curve was produced by plotting the standards (a linear relationship was determined) and the equation of the line was used to calculate the concentration of the samples.

Western blot analysis

Cells were treated with 0, 25, 50 or 75 μM mavacoxib for 24 hours prior to harvesting for western blot analysis. Cells were harvested and cell pellets were lysed with urea lysis buffer (7 M urea, 0.1 M DTT, 0.05% Triton X-100, 25 mM NaCl, 20 mM Hepes pH 7.5). A Bradford assay was used to quantify protein concentrations of samples prior to all western blotting using Quick Start™ Bradford 1x Dye Reagent (Bio-Rad Laboratories, California, USA). Equal amounts of protein (30 μg) were resolved based on their molecular weight by SDS polyacrylamide gel electrophoresis (SDS PAGE) [51], transferred to Amersham™ Hybond-C™ nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridised to an appropriate primary antibody and HRP-conjugated secondary antibody for subsequent detection by enhanced chemiluminescence (Amersham™ ECL™, Amersham Pharmacia Biotech).

The following primary antibodies were utilized to assess protein expression: COX-2 (C-terminus of COX-2 of human origin, sc-1745) from Santa Cruz Biotechnology (Texas, USA); ERK1/2 (p44/42 MAPK (137F5)), phosphorylated-ERK1/2 (phospho-p44/42 MARK, dually phosphorylated at Thr202/Tyr204), Akt (Akt1, Akt2 and Akt3), phosphorylated-Akt (Akt1 phosphorylated at Ser473, and Akt2 and Akt3 when phosphorylated at corresponding residues) all from Cell Signalling Technology (Massachusetts, USA); and β-actin (N-terminal of the beta isoform of actin, ab6276) from Abcam (Cambridge, UK). Secondary antibodies were HRP-conjugated swine anti-rabbit IgG, rabbit anti-mouse IgG and rabbit anti-goat IgG from Dako, Agilent Technologies (CA, USA).

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis of student's t-test or Mann-Whitney test were performed using Minitab® statistical software (PA, USA). Spearman's correlation for nonparametric analysis was performed using GraphPad Prism version 7.0c for Mac OS X (GraphPad software, CA USA). Differences were deemed significant with a p-value of less than 0.05.

Results

Characterization of COX-2 expression and PGE_2 production in a panel of canine and human cancer cell lines

A panel of canine and human cancer cell lines were characterised by cellular morphology and doubling time (Supplementary table 1). We showed a spectrum of morphology from classic cobblestone morphology associated with epithelial cells (for example, the mammary carcinoma cell line REM and the human bladder transitional cell carcinoma cell lines K9TCC, K9TCC-AXA, K9TCC-In and K9TCC-Sh) and elongated protrusions associated with mesenchymal cells (for example, the canine osteosarcoma cell lines CSKOS and KTOSA5 and the inflammatory mammary carcinoma cell line LILY). There was also a range in doubling time from 7.0 (T24 cells) to 10.6 hours (K9TCC-In cells) (Supplementary table 1).

The expression of COX-2 protein in each cell line was determined by western blotting (Figure 1A). COX-2 expression was negligible in U2OS (human osteosarcoma), CSKOS (canine osteosarcoma) and T24 (human bladder TCC) cell lines, whereas low level COX-2 expression was detected in KTOSA5 (canine osteosarcoma), LILY (canine inflammatory mammary carcinoma) and HT-1376 (human bladder TCC) cell lines and high COX-2 expression was detected in REM (canine mammary carcinoma), 5637 (human osteosarcoma) and in K9TCC, K9TCC-AXA, K9TCC-In and K9TCC-Sh (canine bladder TCC cell lines). COX-2 expression was quantified from western blot analysis relative to β-actin for each cell line and this data is displayed in Table 1. One of the main products of the COX-2 enzyme reaction is PGE_2 and we confirmed the amount of PGE_2 produced by each cell line by ELISA (Table 1). We show that bladder TCC cell lines produce more PGE_2 than the osteosarcoma and mammary carcinoma cell lines (Table 1). To determine if there is a correlation between PGE_2 production and COX-2 expression we utilized the Spearman's statistical test due to the nonparametric nature of the data. Here, we confirmed that COX-2 expression and PGE_2 production are positively correlated ($r=0.7478$, Figure 1B) and that this correlation is statistically significant ($p=0.0071$).

Mavacoxib is cytotoxic to human and canine cancer cell lines and induces replicative cell death

To determine if mavacoxib is cytotoxic to cancer cells we treated a panel of cancer cell lines with increasing doses of mavacoxib and assessed cell viability 72 hours after treatment. We showed that cell viability was reduced in a dose-dependent manner by mavacoxib in all cell lines tested indicating that mavacoxib is cytotoxic (Figure 2Ai-v. Data shown is representative of all cell lines). However, sensitivity to mavacoxib varied between

the cell lines, with IC50 values ranging from 34.5 μM (K9TCC-AXA) to 157.7 μM (HT-1376) (Table 2). To determine if there is a correlation between COX-2 expression and sensitivity to mavacoxib, Spearman's statistical test was used to assess correlation between IC50 values and COX-2 expression. There was no statistically significant correlation between these factors ($r = -0.2222$, $p = 0.4839$), suggesting that mavacoxib may be an effective cytotoxic agent against these cell types, including those with low COX-2 expression.

To elucidate the downstream signalling pathways involved in cell survival that may be affected by mavacoxib we treated cells with increasing doses of mavacoxib for 24 hours and probed for ERK, phosphorylated-ERK (p-ERK), Akt and phosphorylated-Akt (p-Akt) (Figure 2B. representative data shown). COX-2 is known to activate the MAPK/ERK and PI3K/Akt pathways by the action of PGE_2 via EP2/EP4 receptors [52–54]. Dual phosphorylation of both ERK and Akt is required for activation, which both then go on to phosphorylate numerous transcription factors downstream that are involved in promoting cell growth and cell cycle progression. Several studies have demonstrated that COX-2 inhibitors induce apoptosis and reduce cell proliferation and survival via the ERK and Akt pathways [55,56]. The response of these pathways to increasing doses of mavacoxib in the cell lines tested here was variable. In CSKOS cells, p-Akt was downregulated with increasing does of mavacoxib, as was total Akt in U2OS cells. In REM cells both p-ERK and p-Akt were increased in expression with increasing doses of mavacoxib, and in K9TCC cells p-ERK expression was also increased with mavacoxib treatment. This result suggests that mavacoxib may inhibit the Akt pathway in canine osteosarcoma cell lines (CSKOS and U2OS) to reduce cell viability. Interestingly, other studies have noted that the activation of the ERK pathway, which is usually associated with enhancing cell proliferation and survival, can induce anti-proliferative effects and that this can occur with the use of COX-2 inhibitors [24,57,58]. Further investigation

into these and other proliferation pathways will be required to determine the downstream mechanisms responsible for the reduction in cell viability caused by mavacoxib in other cell types.

To substantiate the previous data, we determined the effect of mavacoxib on colony forming ability as an assay of replicative cell death. We showed that mavacoxib treatment significantly reduced colony formation in a dose-dependent manner in all cell lines tested (Figure 3A-C). The ability of mavacoxib to induce replicative cell death is comparable between canine and human bladder TCC cell lines, with a dose-dependent decrease in colony forming ability detected across the panel (Figure 3C). The induction of replicative cell death by the COX-2 inhibitor mavacoxib supports previous data demonstrating a reduction in cell viability with treatment of this drug.

Mavacoxib can induce caspase-dependent apoptosis in canine and human cancer cell lines

Cancer cells have the ability to evade apoptosis by deregulating apoptotic pathways, allowing damaged cancer cells to grow rapidly to form an aggressive tumour. Here, we demonstrate that mavacoxib can induce caspase-dependent apoptosis in all cell lines tested when treated at 100 μM or above (Figure 4). A dose-dependent increase in the number of apoptotic cells is detected in KTOSA5, U2OS, REM and K9TCC cells (Figure 4A-C and 4E), however only the highest dose of mavacoxib produced a statistically significant response in LILY (Figure 4D) and T24 cells (Figure 4F). LILY and T24 cells express low levels of COX-2, however KTOSA5 and U2OS cells also express low levels of COX-2 and these cell lines remain more sensitive to mavacoxib induced caspase-dependent apoptosis. Mavacoxib was

able to reduce cell viability in LILY and T24 cells at lower concentrations, suggesting that these cell lines may be inherently resistant to mavacoxib-induced caspase-dependent apoptosis, but other apoptotic pathways may be involved.

Cellular migration is inhibited by mavacoxib in a cell-line dependent manner

The ability of mavacoxib to inhibit the migration of human and canine cancer cell lines *in-vitro* was investigated (Figure 5). Either a standard scratch assay (KTOSA5, U2OS, REM and LILY cell lines (Figure 5Bi-iv)) or the Ibidi® Culture-Insert migration assay (K9TCC and T24 cell lines (Figure 5 Bv-vi)) was used to measure the migration of cancer cells incubated with mavacoxib for 24 hours. Here, we demonstrate that mavacoxib reduces the migration of human and canine cancer cell lines *in-vitro* (Figure 5B). The lowest concentration of drug to yield a statistically significant result is shown for each cell line, with a representative image of the assay conducted in K9TCC cells shown in Figure 5A. The canine and human osteosarcoma cell lines (KTOSA5 (Figure 5Bi) and U2OS (Figure 5Bii) respectively) respond in a comparable manner, however the human bladder TCC cell line (T24) requires a higher concentration of mavacoxib to yield a significant result (Figure 5Bvi) compared to the canine bladder TCC cell line (K9TCC) (Figure 5Bv). The inflammatory mammary carcinoma cell line LILY required higher doses of mavacoxib and a larger initial gap width before a significant response was detected, consistent with this cell line being more resistant to mavacoxib (Figure 5Biv). These results indicate that mavacoxib inhibits cell migration in cell-line dependent manner.

Discussion

This study highlights a novel use for mavacoxib as a potential anti-cancer agent in canine patients. The link between inflammation, COX-2 and cancer is well established; when inflammation and oncogenic activation converge, inflammatory transcription factors are activated in tumour cells, which produce inflammatory mediators, cytokines and chemokines, including COX-2, that recruit and activate inflammatory immune cells. These cytokines activate the same key transcription factors in inflammatory cells, tumour cells and stromal cells, resulting in a surge of inflammatory mediators giving rise to a cancer related inflammatory microenvironment [59,60]. This smouldering inflammatory environment has many tumour promoting effects, including enhancing proliferation and survival, promoting EMT, metastasis and invasion, encouraging angiogenesis and lymphangiogenesis, inhibiting the adaptive immune response and altering responses to hormones and chemotherapeutic agents [60,61].

This study demonstrates that mavacoxib is able to reduce cancer cell survival and induce cell death. To further understand the mechanisms behind this, the activation of ERK and Akt were investigated after cell treatment with mavacoxib. We demonstrate a variable response in the phosphorylation status of both ERK and Akt after treatment with mavacoxib, with phosphorylation increasing with treatment in some cell lines (REM and K9TCC) and decreasing in others (CSKOS and U2OS). Generally, it has been shown that the inhibition of COX-2 decreases phosphorylation of both ERK and Akt which in turn inhibits proliferation, induces apoptosis and results in cancer cell death. However, several studies have also demonstrated that inhibition of COX-2 increases the phosphorylation of these molecules and incongruously, this increase in phosphorylation was also associated with apoptosis [24,57,58,62]. Various NSAIDs have been shown to induce changes in ERK phosphorylation between 4 and

24 hours of incubation [24,62,63]. Yip-Schneider et al (2003) discuss that short term incubation (1 hour) of pancreatic carcinoma cells with suldinac did not induce changes in phosphorylation but 24-hour incubation did, suggesting that long-term rather than transient activation of this pathway was occurring. The basal activation level of the ERK and Akt pathways may also influence the extent to which cells may respond in this way to NSAIDs [62]. Therefore, the response of each cell line to the ERK / Akt phosphorylation effects of NSAIDs may be time and cell type dependent. Future studies should include cell lines with low and high basal activation of these pathways incubated with various different NSAIDs for various times in order to investigate what causes the active or suppressive response.

Mavacoxib can induce caspase-dependent apoptosis in a number of cell lines tested here (Figure 4). This occurs at higher drug concentrations than the reduction in cell viability, suggesting at lower concentrations, mavacoxib may induce caspase-independent cell death mechanisms. Caspase-independent cell death has been previously characterised, highlighting that caspase inhibitors do not prevent cell death through autophagy, necrosis and apoptosis-like cell death [64]. Previous studies have noted similar results with NSAIDs in cancer cell lines, with caspase-independent and BAX-independent apoptosis suggested as a possible explanation [35,37,65]. We have previously shown that increased Bcl-2 levels after COX-2 inhibition may be the result of a mitochondrial-independent apoptotic pathway [35]. Further investigation into the mechanisms driving mavacoxib-induced cell death should include methods able to detect early stage apoptosis that may be caspase-independent.

The survival rate of patients with metastatic cancer is greatly reduced in comparison to those with a primary tumour. COX-2 has been implicated in the process of epithelial to mesenchymal transition (EMT), which allows epithelial primary tumour cells to gain invasive

and metastatic potential by converting to a mesenchymal phenotype with enhanced mobility [66]. Several studies have established potential for utilizing COX-2 inhibitors to induce mesenchymal to epithelial transition (MET), thereby inhibiting migration and invasion of cancer cells. In human bladder cancer, the T24 cell line exhibits a strong EMT phenotype which can be reversed by the COX-2 inhibitor etodolac [67]. Che et al. (2017) have demonstrated in lung cancer cells that migration and invasion are stimulated via interleukin-6 (IL-6) upregulation of COX-2/PGE_2 [68], with a similar finding in head and neck squamous cell carcinoma [69]. In this study, we report that mavacoxib can inhibit migration of a panel of canine and human cancer cell lines and that this action is cell line dependent. To establish whether mavacoxib exerts its anti-migration effects by reversing EMT, future studies should determine the expression of E-cadherin in each cell line before and after mavacoxib treatment, along with expression of EMT transcription factors and mesenchymal markers.

Interestingly, the LILY cell line migrated faster and was more inherently resistant to mavacoxib than the other canine mammary carcinoma cell line tested. The LILY cell line was derived from a canine inflammatory mammary carcinoma, a fast growing, highly malignant cancer type in which COX-2 has been shown to be overexpressed with increased risk of metastasis and lymph node invasion [70–72]. Here, we suspect an inflammatory response by the LILY cell line, whereby the act of scratching a confluent layer of inflammatory mammary carcinoma cells with a pipette tip results in an apoptosis induced inflammatory response that stimulates cell proliferation and migration. There is increasing evidence that cells undergoing apoptosis signal their presence to the surrounding tissues to elicit tissue repair and regeneration [73]. This phenomenon of tissue damage initiating tissue repair has been coined the “phoenix rising pathway” [74] and a seminal study in bladder cancer has demonstrated evidence for the involvement of the COX-2/PGE_2 pathway in cancer stem cell (CSC)

repopulation of residual tumours in response to chemotherapy-induced damages [75]. Further investigation is required to assess apoptotic activity after damage induction in the LILY cell line, along with the expression of COX-2 and release of PGE_2.

Many NSAIDs and COX-2 selective inhibitors have demonstrated COX-2 independent anti-cancer effects. Here, we show that mavacoxib can exert anti-tumourigenic effects on cell lines with low COX-2 expression, advocating that mavacoxib may exert some of its anti-tumour effects in a COX-2 independent manner. Aside from their COX-inhibitory function, NSAIDs have a demonstrated a number of properties that make them attractive anti-cancer therapeutics, including anti-proliferative and pro-apoptotic activity [76], anti-thrombotic [77] and anti-angiogenic effects [78]. Potential mechanisms of these non-COX-2 inhibitory actions include the inhibition of carbonic anhydrase and polyamines. For example: De Monte et al. (2015) discuss the ability of sulphonamide COX-2 selective inhibitors to inhibit several carbonic anhydrase isoforms, enzymes which are important in cancer cell survival in hypoxic environments [79]; and indomethacin, a NSAID, has also been shown to increase the catabolism of polyamines, regulators of tumour cell proliferation, by upregulating the activity of spermidine/spermine-acetyltransferase (SSAT) in human colon cancer cell lines [80]. Interestingly, derivatives of NSAIDs that lack the ability to inhibit the COX enzymes retain their anti-tumour activity [81–83] and these COX-independent targets have been associated with apoptotic, proliferative, angiogenic and metastatic pathways [81] but have yet to be fully elucidated. An interesting future research avenue would be to confirm the COX-2-dependent and COX-2-independent effects of mavacoxib by producing a mavacoxib derivative that lacks COX-2 inhibitory action, and to determine whether mavacoxib inhibits the carbonic anhydrase or polyamine pathways.

Currently, there are a number of NSAIDs approved for clinical use. With ample epidemiological evidence demonstrating an inverse correlation between NSAID use and the incidence and progression of cancer, there have been numerous trials attempting to validate these drugs for use as cancer therapeutics. Aspirin, celecoxib, ibuprofen and piroxicam have all demonstrated positive anti-neoplastic effects during clinical trials [76,84–90]. However, the use of these drugs for cancer prevention and therapy remains controversial. Many clinical studies have not indicated a significant benefit to the patient, and in combination with the side-effects associated with NSAIDs (which can range from moderate GI toxicity to severe cardio toxicity), there is limited enthusiasm for their use [91]. Given these controversies it is unlikely that COX-2 inhibitors in particular will be utilized as a monotherapy for cancer, however, they may be effective adjuvants when used in conjunction with other therapeutic approaches. For example, dual modality treatments are currently offered for several human cancer types, including colorectal and prostate cancer [92–94].

In the field of veterinary oncology, numerous trials are attempting to elucidate the potential of NSAIDs as a cancer treatment for dogs, both as a single treatment and as part of dual modality or metronomic chemotherapy treatment options. Piroxicam, a non-selective COX inhibitor used in human medicine, has been one of the most widely studied. Piroxicam as a single mode treatment has been established as an effective anti-cancer agent in the treatment of transitional cell carcinoma of the urinary bladder in dogs, with cases of both complete and partial remission noted [95]. In canine prostatic carcinoma, both piroxicam and carprofen (a preferential COX-2 inhibitor; this class of drugs affects both COX-1 and COX-2 but is more selective towards COX-2) significantly increased survival time as single agents when compared to untreated dogs in both early stage and metastatic disease [96]. As part of dual modality treatment and metronomic chemotherapy, piroxicam significantly increased

survival time and incidences of complete or partial remission compared to the same treatments without piroxicam in a range of cancer types [97] including invasive urothelial carcinoma [98] and primary lung carcinoma [99]. A recent review on canine mammary carcinoma concludes that including NSAIDs in treatment significantly improves survival, especially in inflammatory mammary carcinomas when NSAIDs are used alone as palliative treatment or in combination with chemotherapy [100]. However, conflicting evidence still remains. Studies in transitional cell carcinoma of the urinary bladder, nasal carcinomas and multicentric lymphoma utilizing combinations of chemotherapeutics or radiotherapy with piroxicam and firocoxib demonstrated no significant difference in progression free and overall survival compared to other treatment regimens [101–103]. A study in mammary carcinoma demonstrated that surgery plus adjuvant chemotherapy significantly improved overall survival time compared to surgery alone, but there was no significant difference when chemotherapy was combined with or without NSAIDs [104]. COX-2 expression was found to be a significant factor in survival time in this study and the author suggests that immunohistochemical score of COX-2 should be included as a predictive factor for this cancer type [104]. Interestingly, Knapp et al. (2016) noted that when vinblastine treatment was followed by piroxicam as two single agents there was a significant increase in overall survival time compared to dogs receiving the same agents simultaneously. It is suggested that this could be the consequence of drug resistance developing separately for each drug over time rather than concurrently, which raises the interesting possibility that chemotherapy may sensitize the tumour to the action of COX inhibitors [98]. After chemotherapy treatment, resistant cells can go on to repopulate the tumour, a process that has been shown to involve the COX-2 pathway [75]. Therefore, chemotherapy may select for a clone of tumour cells with increased COX-2 activity, rendering them more sensitive to the actions of COX inhibitors. If this occurs, even

tumours with low COX-2 expression initially may benefit from a treatment program consisting of chemotherapy and NSAIDs sequentially.

One of the recurring topics across many of the studies mentioned above is that of quality of life, a primary concern when treating veterinary cancer patients. In many cases curative treatment is not an option and palliative care is opted for. In the studies mentioned above, several factors used to assess quality of life (including appetite, activity levels and pain) were all noted to be improved in patients treated with NSAIDs (reported through owner questionnaires), even in cases where NSAIDs showed no improvement in survival time compared to other treatment options [95,99,101,102]. In one study, most owners requested that their dogs remain on piroxicam treatment even if tumour remission did not occur, due to their subjective improvement in quality of life [95]. Given the importance of quality of life, this is clinically relevant finding and should be taken into consideration when decisions are made regarding treatment options in veterinary cancer care.

Currently the use of mavacoxib in the clinic is unorthodox as a result of the long half-life of the drug potentially resulting in side-effects which are problematic for the patient. The usual dosing regimen for mavacoxib comprises of administration at monthly intervals after initial doses of 2 mg/kg at day 0 and day 14, maintaining plasma concentrations at a steady state of around 0.52 – 1.11 μg/μl (1.35 – 2.88 μM) [105,106]. Studies have demonstrated that the safety profile of mavacoxib is comparable to other veterinary NSAIDs [107,108], and suggests that the benefits of reduced dosing should be exploited. Although the concentrations of mavacoxib utilized in this study are higher than plasma concentrations, there is currently a concerted research effort to develop targeted drug delivery systems for cancer therapies which would allow increased concentrations of therapeutic agents to target the tumour site

while minimising undesirable side-effects. These include using nanoparticles to encapsulate therapeutic agents and release them in a controlled manner to target diseased cells with increased specificity [109–111]. If the challenges of dosing and toxicity for drugs like mavacoxib can be diminished by improved drug delivery systems, their anti-cancer potential may be exploited.

In conclusion, we show that mavacoxib is an effective cytotoxic agent on a range of tumour types and across species. We highlight that mavacoxib can exert anti-tumour effects on cells expressing a low level of COX-2, however further mechanistic studies are required to understand the COX-2 dependent and COX-2 independent actions of mavacoxib. Further *in vivo* and preclinical studies will be required to fully elucidate the potential of mavacoxib as an anti-cancer agent in conjunction with its clinical benefits as an anti-inflammatory drug.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Tables

Table 1. COX-2 expression and PGE_2 production in a panel of canine and human cancer cell lines.

Cell line	COX-2 expression	PGE_2 production (ng/mL)
U2OS	0.00	2.44
KTOSA5	0.24	2.32
CSKOS	0.00	2.14
REM	0.51	2.45
LILY	0.36	2.61
K9TCC	0.58	5.54
K9TCC-AXA	0.99	5.44
K9TCC-In	0.99	5.67
K9TCC-Sh	0.76	6.17
T24	0.00	5.23
5637	0.77	5.83
HT-1376	0.62	6.14

COX-2 expression was quantified by densitometry relative to β-actin expression. PGE_2 production was assessed by ELISA using conditioned media from cells incubated for 24 hours in fresh cell culture media.

Table 2. The IC50 was calculated for each cell line treated with mavacoxib.

Cell line	IC50 mavacoxib [µM]
U2OS	52.6
KTOSA5	89.8
CSKOS	106.3
REM	66.6
LILY	97.5
K9TCC	54.9
K9TCC-AXA	34.5
K9TCC-In	78.7
K9TCC-Sh	50.7
T24	63.4
5637	72.5
HT-1376	157.7

IC50 values were calculated for each cell line based on response in the cell viability assay (Figure 2).

Figure legends

Figure 1. COX-2 expression and PGE_2 production of a panel of canine and human cancer cell lines. (A) COX-2 expression was assessed for each cell line by western blot analysis. 30 µg of protein was loaded per well and β-actin was used as a loading control. (B) Spearman's nonparametric statistical test was used to assess correlation between COX-2 expression and PGE_2 production. Spearman's correlation (r = 0.7478) determined that COX-2 expression and PGE_2 production are positively correlated and this correlation is statistically significant (p=0.0071 **).

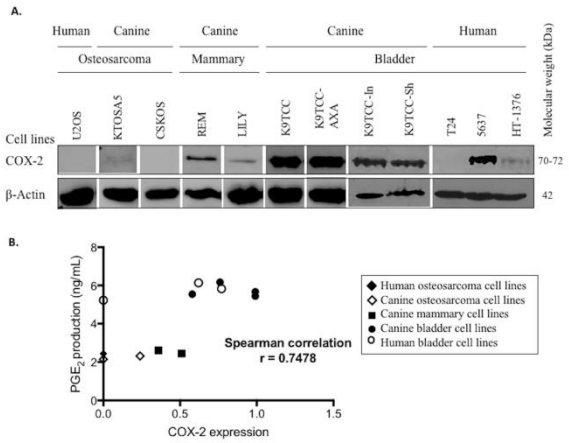


Figure 2. Mavacoxib reduces cell viability in a dose-dependent manner. **(A)** All cell lines were subject to increasing doses of mavacoxib and incubated for 72 hours prior to measuring cell viability. Data is shown for two osteosarcoma cell lines (canine CSKOS (i) and human U2OS (ii)), one canine mammary carcinoma cell line (REM (iii)) and two bladder TCC cell lines (canine K9TCC (iv) and human T24 (v)). Differences were deemed significant with a p-value of less than 0.05 ($p<0.05$ *, $p<0.01$ **, $p<0.001$ #). **(B)** Downstream markers of proliferation were analysed by western blot analysis after treatment with mavacoxib. Cells were subject to increasing doses of mavacoxib for 24 hours prior to harvesting and probed for COX-2, ERK, phosphorylated-ERK (p-ERK), Akt and phosphorylated-Akt (p-Akt). 30 µg of protein was loaded per well and β-actin was used as a loading control.

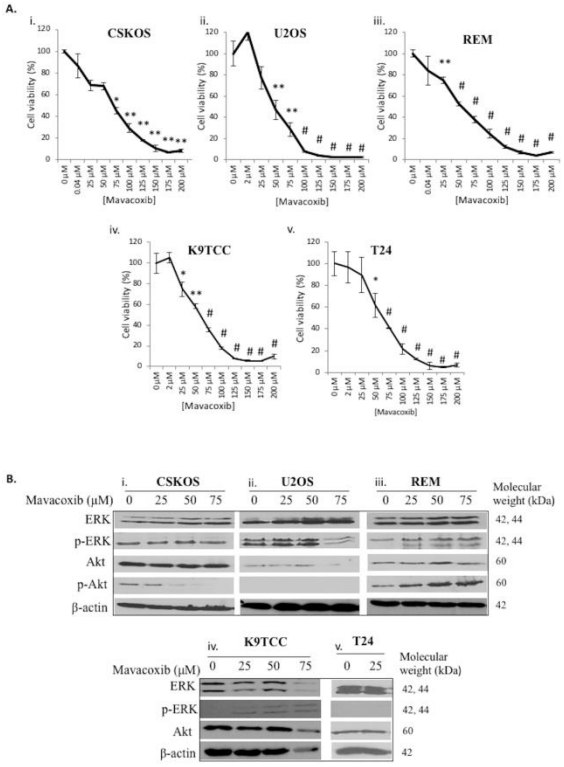


Figure 3. Mavacoxib induces replicative cell death in a panel of canine and human cancer cell lines. The colony forming ability of (**A)** osteosarcoma cell lines (canine CSKOS and KTOSA5 and human U2OS), **(B)** canine mammary carcinoma cell lines (REM and LILY) and **(C)** bladder TCC cell lines (canine K9TCC, K9TCC-Sh, K9TCC-In, K9TCC-AXA and human 5637, T24 and HT-1376) was assessed with increasing doses of mavacoxib. After 14 days, the number of colonies were counted manually. Differences were deemed significant with a p-value of less than 0.05 ($p<0.05$ *, $p<0.01$ **, $p<0.001$ #).

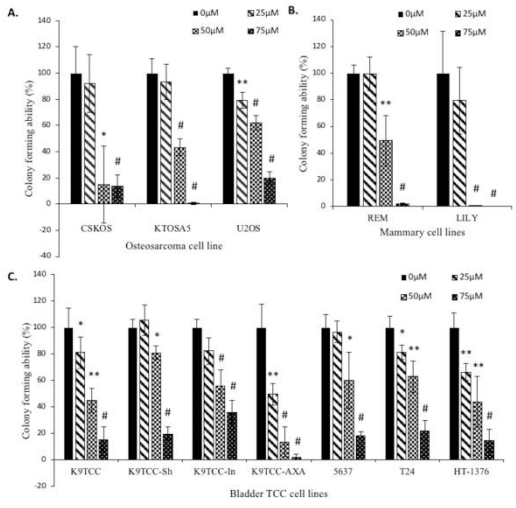


Figure 4. Mavacoxib induces apoptosis in canine and human cancer cell lines. A panel of cancer cell lines (canine osteosarcoma KTOSA5 **(A**), canine mammary REM **(C**) and LILY **(D**), canine bladder TCC K9TCC **(E),** human osteosarcoma U2OS **(B)** and human bladder TCC T24 **(F)**) were subject to increasing doses of mavacoxib and apoptotic activity was measured 48 hours after treatment. Differences were deemed significant with a p-value of less than 0.05 ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***).

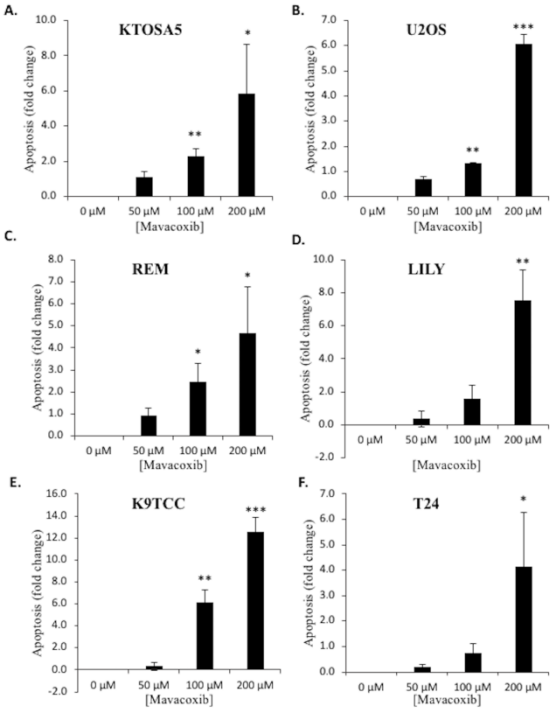
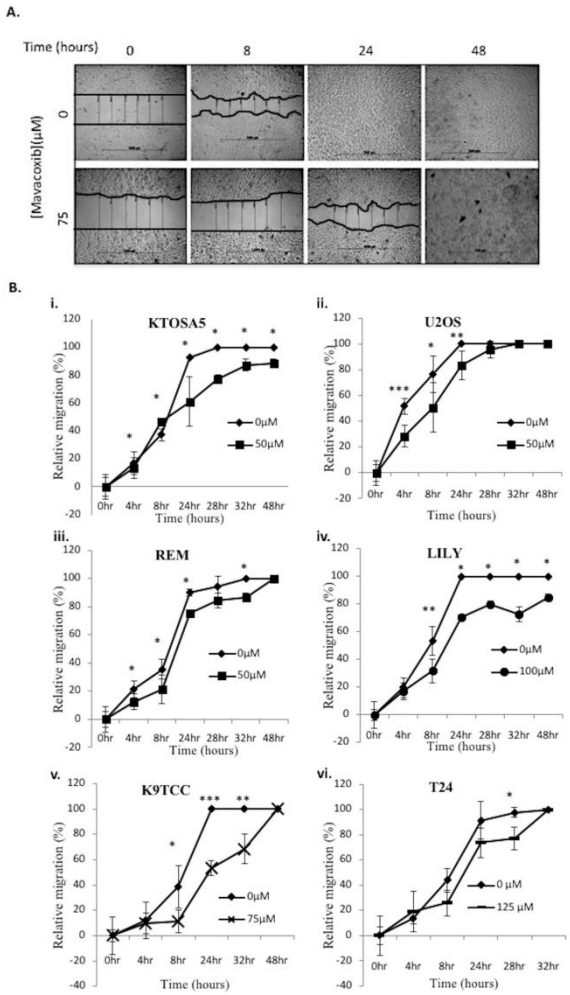


Figure 5. Mavacoxib inhibits cellular migration in a cell-line dependent manner. **(A)** Representative image is of the Ibidi® migration assay. Canine bladder TCC K9TCC cells were seeded into each well of the insert and treated with mavacoxib. After 24 hours incubation, the insert is removed and the gap distance is measured at several time points up to 48 hours. Six points were measured along the gap and the average distance calculated at each time point. **(B)** A panel of canine osteosarcoma (KTOSA5 (i)), canine mammary (REM (iii) and LILY (iv)), canine bladder TCC (K9TCC (v)), human osteosarcoma (U2OS (ii)) and human bladder TCC (T24 (vi)) cancer cell lines were treated with mavacoxib and cellular migration was assessed. Shown are the lowest doses of mavacoxib able to induce a

statistically significant response in each cell line. Differences were deemed significant with a p-value of less than 0.05 ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***).



Supplementary files

Supplementary table 1. Panel of canine and human cancer cell lines. The panel of canine and human cell lines utilised in this study, highlighting the species and cancer type. Each cell line was characterised by cellular morphology and doubling time.